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Quantification of Infectious Duck Hepatitis B Virus by Radioimmunofocus Assay

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A simple method is described for the precise quantification of infectious duck hepatitis B virus (DHBV) in cell culture, using a radioimmunofocus assay (RIFA). Primary duck hepatocyte cell cultures were infected with serial dilutions of viral samples as for a plaque assay, but then maintained with liquid overlay medium. After incubation for up to 14 days, cell monolayers were fixed with acetone, then stained with a monoclonal antibody to DHBV L protein followed by secondary antibody labelled with ^{125}I. Foci of infection (representing individual infectious particles in the inoculum) were detected by autoradiography. The number of foci recovered was increased by addition of dimethyl sulphoxide to culture medium, but was not appreciably altered by the use of semi-solid medium. The titre of virus suspensions determined by RIFA correlated well with titration in ducklings. The RIFA is a useful method for titration of DHBV, as it has a wide dynamic range and is well suited to parallel titration of large numbers of samples. This assay will have wide use for the analysis of DHBV growth kinetics, antiviral efficacy, and virus inactivation procedures. ***J. Med. Virol. 52:354–361, 1997.***

KEY WORDS: DHBV; infectivity assay; viral quantification

INTRODUCTION

The accurate quantification of infectious viral titres is a cornerstone of virology which has been problematic for the hepatitis viruses due to their limited replication in cell culture, most often without significant cytopathic effects. The development of a radioimmunofocus assay (RIFA) for hepatitis A virus (HAV) by Lemon et al. [1983] was a major technical advance, as it allowed the detection of foci of infection in a "plaque" by indirect methods in the absence of viral cytopathic effects. This assay technique has since been used effectively for other non-cytopathic viruses such as human rotaviruses [Liu et al., 1984], and remains the method of choice in most laboratories studying HAV.

Duck hepatitis B virus (DHBV) is used widely as a model for studies of the *Hepadnaviridae* family, as this virus can be readily propagated in primary duck hepatocyte cell cultures whereas the mammalian hepadnaviruses (including human hepatitis B virus) are difficult or impossible to culture. Although cytopathic mutants have been described [Lenhoff and Summers, 1994], wild-type DHBV is noncytopathic and a number of methods have therefore been reported for the determination of infectious virus titres. Quantitative assays have been described based on detection of foci by indirect immunofluorescence [Pugh and Simmons, 1994; Pugh and Summers, 1989], but this is inconvenient for large numbers of samples. Quantal assays can be performed in either cell cultures [Lambert et al., 1991] or live ducks [Murray et al., 1991] with endpoints determined by hybridisation of viral DNA. Simple, semi-quantitative assays have proved useful, based on the yield of antigen [Klingmuller and Schaller, 1993] or most commonly viral DNA [Cheung et al., 1989; Jilbert et al., 1992; Lenhoff and Summers, 1994; Prince et al., 1993; Pugh et al., 1988] from infected cell cultures. However, these assays are limited by a narrow dynamic range and may be further complicated when used for the analysis of viral mutants with varying replicative efficiencies. Perhaps as a consequence of these limitations, levels of infectious virus are infrequently reported or determined despite extensive studies of this hepadnavirus.

We have therefore applied the basic principles of the HAV RIFA to DHBV grown in primary hepatocyte cultures, and report the development and optimisation of a simple, sensitive and quantitative assay for infec-

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tious DHBV in vitro which should prove useful for studies of this model hepadnavirus.

MATERIALS AND METHODS

Cells and Virus

Pekin-Aylesbury ducklings known to be negative for DHBV were obtained from a commercial supplier and primary hepatocytes were prepared by collagenase perfusion at 5–7 days of age according to a modification [Bishop et al., 1990] of the method of Tuttleman et al. [1986]. Hepatocytes (2 ml at 1.5×10^6 per ml) were plated in six-well (30 mm) multiplates (Greiner, Solingen, Germany) which contained 25 mm "Thermanox" tissue culture coverslips (Nunc, Naperville, IL), and were incubated overnight at 37°C. Medium was L-15 with normal supplements [Tuttleman et al., 1986] plus 1% dimethyl sulphoxide (DMSO) [Galle et al., 1989; Pugh and Summers, 1989] and without fetal calf serum (FCS).

One day after plating, cells were infected with 0.4 ml of serial 10-fold dilutions of virus in L-15 plus 1% FCS, for 2 hr at 37°C. Cultures were then maintained in L-15 plus 1% DMSO for 12 days before removal of coverslips from the well, and fixing of cell monolayers in cold acetone for 2 min. SeaPlaque agarose (0.5%; FMC Bioproducts, Rockland, MD) or suramin (100 μg/ml; Calbiochem, San Diego, CA) [Petcu et al., 1988] were added at 2 days post-infection where shown to restrict the spread of virus in the culture.

For the preparation of virus stocks, DHBV-free 1-day-old ducklings were infected via intraperitoneal injection with 0.5 ml of serum collected from a single congenitally infected duck (obtained from a separate commercial supplier). Serum was collected 3 weeks (pool 1, used for all experiments except where indicated) and 4 weeks later (pool 2), and stored in aliquots at −70°C.

Monoclonal Antibodies to DHBV Proteins

Monoclonal antibody (MAb) preS1 [Luscombe et al., 1994] was a gift of Scott Bowden, Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia. MAb 1H.1, also specific for the preS domain of L, was a gift of John Pugh, Fox Chase Cancer Centre, Philadelphia, PA. Samples were incubated with hybridoma cell culture supernatants at a dilution of 1:200 in phosphate buffered saline (PBS, pH 7.4) containing 2% FCS. For indirect immunofluorescence, fluorescein-labelled anti-mouse IgG (Dako, Glostrup, Denmark) was used at a dilution of 1:200 in the same buffer.

Detection of Infectious Foci

Acetone-fixed monolayers were incubated with MAb (0.4 ml) by inversion of the coverslip on drops of diluted hybridoma supernatants, washed in PBS, and foci of infection were detected by counterstaining as above with 0.4 ml of ^{125}I-labelled goat anti-mouse IgG (ICN Biomedicals, Costa Mesa, CA) diluted 1:1,000 in PBS plus 2% FCS. After a further three washes, coverslips were dried and exposed to Fuji RX film with a single intensifying screen at −70°C for 3 to 7 days. The infectious titre in radioimmunofocus-forming units (RIFU) per ml was calculated from the number of foci detected and the dilution, multiplied by a factor of 3.6 to allow for the volume of the inoculum per well (0.4 ml) and the relative areas of the coverslips (4.9 cm^2) and the culture wells (7.1 cm^2) since the remaining cell monolayer is expected to contain foci.

Titration of Infectious Virus in Live Ducklings

Four 1-day-old ducklings were inoculated via intraperitoneal injection with 1.0 ml of virus in L-15 for each dilution. Sera were collected at 4 weeks post-inoculation and assayed for viral DNA using digoxigenin-labelled probes as previously described [Guo and Bow-

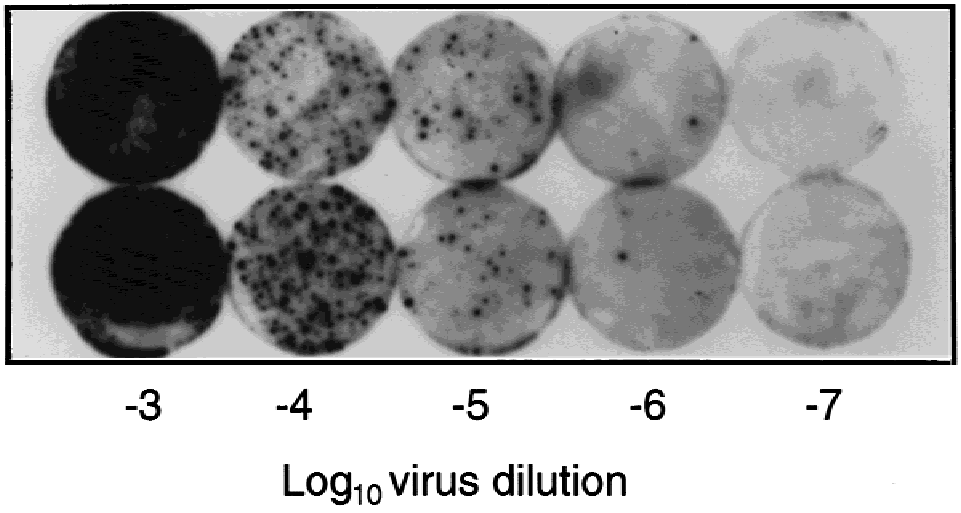

Fig. 1. Radioimmunofocus assay for infectious DHBV. Primary duck hepatocyte cultures were infected with serial dilutions of viraemic duck serum as indicated and were maintained for 12 days, fixed and stained for DHBV L protein, and foci detected by autoradiography.

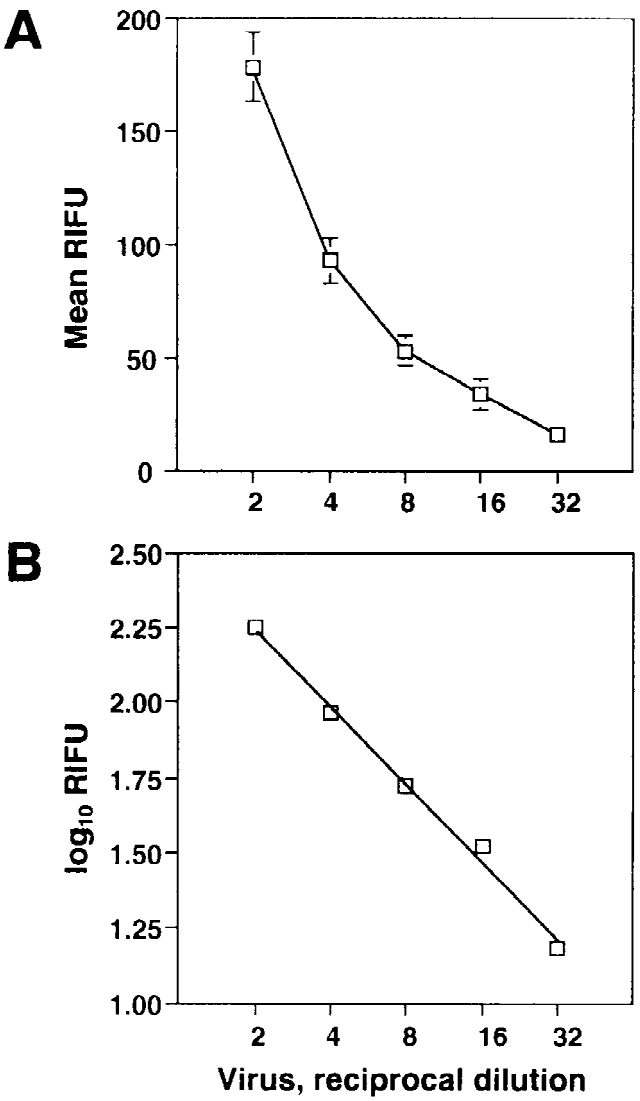

Fig. 2. Dose-response for the development of DHBV foci. Cells were infected and processed as described in Materials and Methods, and foci of infection detected by autoradiography. Numbers of foci from triplicate samples (**A**: mean ± standard deviation; **B**: $\log_{10}$ of mean) were plotted against reciprocal dilution. Note the linear dose-response and one-hit kinetics of the assay.

den, 1991]. The ID_{50} was determined according to the method of Reed and Muench [1938].

RESULTS

Quantitative Detection of Infectious DHBV by RIFA

In our initial experiments, foci of infection representing individual infectious viruses in the inocula were detected readily after 12 days incubation with liquid media and showed an apparently dose-dependent relationship (Fig. 1). This relationship was further examined using a narrow dilution range with a different virus pool (Fig. 2). The numbers of foci recovered at each dilution showed a low variation between replicates (Fig. 2A, showing the mean and standard deviation of assays carried out in triplicate) and confirmed that development of foci followed one-hit kinetics (Fig. 2B), as expected for a quantitative assay. The DHBV RIFA can therefore be considered analogous to a standard plaque assay. In our hands, between five and approximately 180 individual foci per coverslip can be counted reliably, and samples are plated at dilutions estimated to fall in this range.

Time Course for the Development of Foci

Replicate cultures infected with serial dilutions of a DHBV virus pool were fixed at 8, 10, 12, and 14 days post-infection, and stored at −20°C until staining. In the example shown in Figure 3, foci were detectable at 8 days and increased in size (but not number) at 10 days, with no further increase thereafter. However, in some experiments a 12 day incubation gave more clearly defined foci than at 10 days (results not shown), and we now incubate the assay routinely for 12 days.

Foci Develop Through Spread of Virus to Adjacent Cells

One possible limitation of the method described is the need to maintain primary cells for 10 to 12 days, since in our hands semi-solid medium does not always support survival of hepatocytes over this period (results not shown). We therefore examined whether the development of discrete foci proceeded via spreading to adjacent cells or alternatively through release of virus to the supernatant, which might lead to secondary infection and increased numbers of foci. Indirect immunofluorescence of PDH monolayers infected and maintained in liquid media for 12 days showed the development of discrete, large foci with only occasional "satellite" infected cells (Fig. 4), suggesting that spread is predominantly from cell to neighbouring cell. We then examined the effect of suppressing virus spread by the omission of DMSO or the use of semi-solid medium (addition of SeaPlaque agarose) or addition of suramin [Petcu et al., 1988] with cells being fixed after 12 days incubation (Fig. 5). The omission of DMSO resulted in small foci, while cells survived poorly under agarose but produced similar numbers of foci. In the presence of DMSO foci were quantitated easily, while in the presence of suramin few discrete foci could be detected, presumably because this drug prevents spread of virus from the original infected cells [Petcu et al., 1988]. It is not clear whether the few foci detected with suramin represent variants resistant to the drug or background staining, which is occasionally evident in mock-infected monolayers and at the perimeter of some coverslips (for example, cultures at the 10^{-7} dilution maintained under SeaPlaque). As virus spread is predominantly from cell to neighbouring cell even in liquid media, and occasional individual infected cells cannot be detected as foci by autoradiography (for example, the suramin-treated cultures in Fig. 5), the use of liquid overlay is justified for this plaque-type assay.

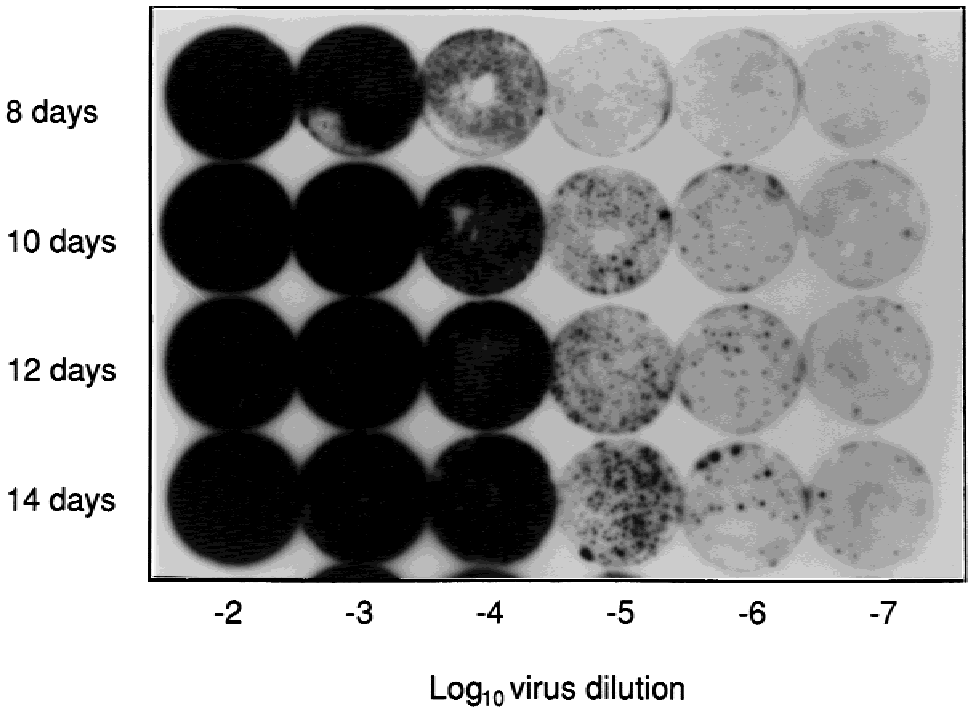

Fig. 3. Time course for the development of visible foci. Replicate cultures infected with serial dilutions of DHBV were fixed at the indicated times post-infection and stored at −20°C until staining. Note that foci increased in size but not number between 8 and 10 days, and no satellite foci were evident at 12 or 14 days.

Cell Seeding Density Affects the Development of Foci

It has been reported that unrestricted spread of hepatocytes on solid substrates decreases their level of differentiation and hepatocyte-specific gene expression [Singhvi et al., 1994]. In addition, as virus spread is from cell to neighbouring cell it was reasoned that development of detectable foci might be dependent on cells making close contact with sufficient neighbours. The influence of cell seeding density on the development of foci was therefore investigated by infection of cultures seeded with 3, 2, or 1 million cells per 3 cm well, with cultures fixed at 12 days post-infection. As shown in Figure 6, development of foci was indeed most efficient at high cell densities (2–3 × 10^6 per well). However, seeding at still higher densities (4 or 6 × 10^6 per well) did not increase the efficiency of the assay, with most cells detaching (results not shown).

Sensitivity of the RIFA Compared to DNA Yield Assay

A very simple method for the semi-quantitative assay of infectious DHBV based on the yield of viral DNA recovered from infected cultures has been reported [Pugh et al., 1988]. We therefore examined the relative sensitivity of the DNA yield and RIFA assays. Dot blot hybridisation was carried out as described previously using ^{32}P-labelled DNA probes [Bishop et al., 1990], giving a detection limit of approximately 1 pg viral DNA. Detection of viral DNA, extracted from replicates of the cultures shown in Figure 6, is shown in Figure 7. DNA hybridisation was not quantitated, but visual examination suggests that the assay response was roughly linear in the range of 10^{-2} to 10^{-5} dilutions of viral inoculum (the limit of detection) at a seeding density of 3 million hepatocytes per well. However, the parallel RIFA detected >100 and 20 foci at 10^{-5} and 10^{-6} dilutions, respectively, indicating a greater sensitivity in the RIFA with a titre of 7.2 × 10^7 RIFU per ml. Although the DNA assay could be made somewhat more sensitive by longer exposure of blots to film, we believe that the quantitative RIFA offers significant advantages over the quantal DNA assay at the limit of viral DNA detection.

Sensitivity of the RIFA Compared to Titration In Vivo

Pekin-Aylesbury ducklings are exquisitely sensitive to infection with DHBV, with samples containing 1 vge being sufficient to initiate infection [Jilbert et al., 1996]. Aliquots of the inoculum described in Figures 6 and 7 were subjected to endpoint titration in ducklings, with the development of viraemia assessed at 4 weeks postinoculation by dot blot hybridisation (Table I). The ID_{50} of this inoculum was found to be 5.4 × 10^8 per ml, close to our estimate of 1.5 × 10^8 vge per ml by dot blot hybridisation (results not shown) and somewhat higher than the RIFA (7.2 × 10^7 per ml). These data indicate that the RIFA is only marginally less sensitive than titration in live ducklings, with the dose for 1 RIFU approaching 8 ID_{50}. Although it appears that our esti-

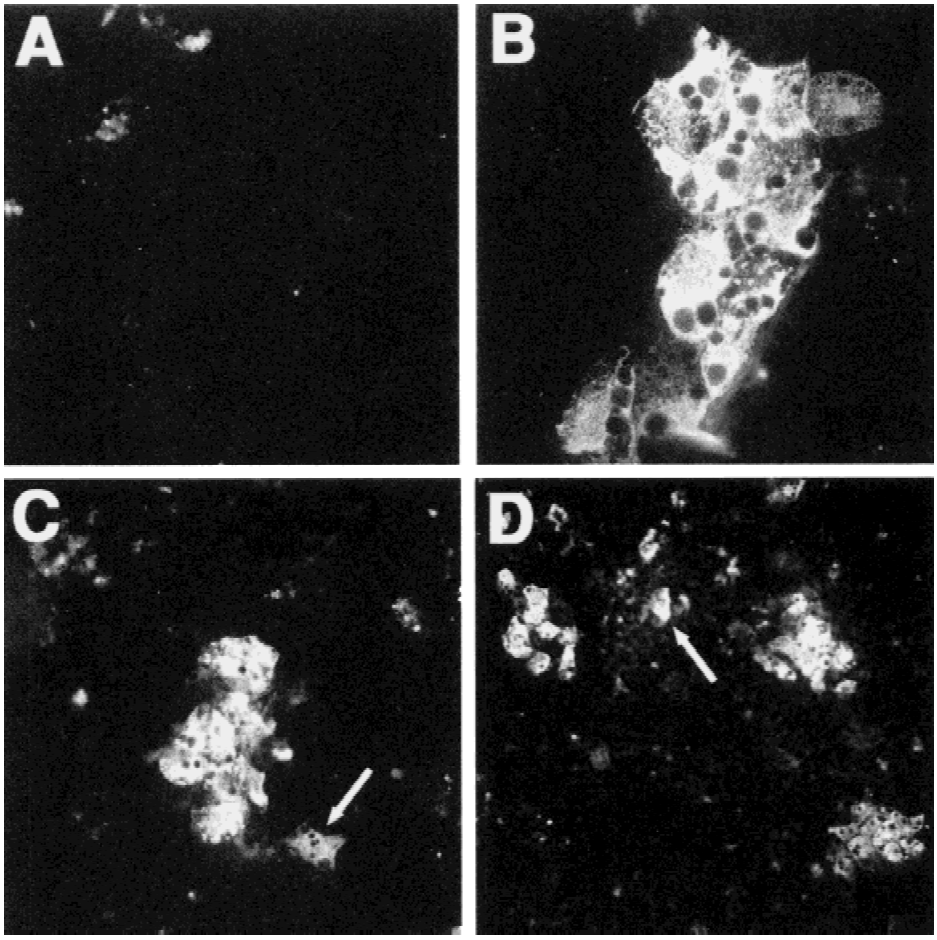

Fig. 4. Indirect immunofluorescence of primary duck hepatocytes fixed at 12 days post infection. **A**: Mock-infected cells. **B–D**: Foci in DHBV-infected cells. Isolated infected cells, probably representing secondary spread beyond foci, are arrowed in C and D. Original magnifications were 400× (B), 200× (C), or 100× (A,D).

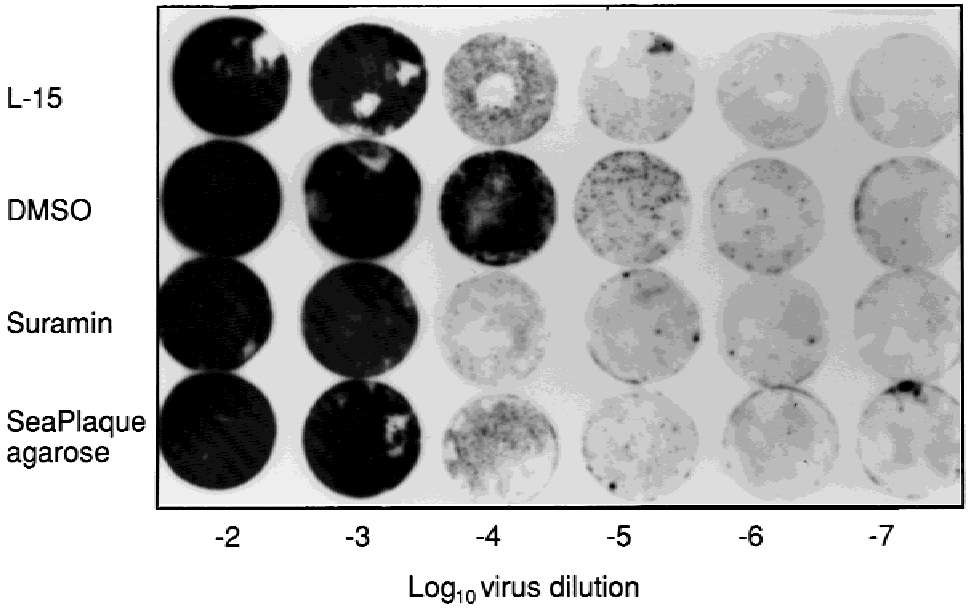

Fig. 5. Development of foci is influenced by additives to the medium. Replicate infected cultures were maintained for 12 days with L-15 or L-15 containing DMSO (1%), suramin (100 μg/ml) or SeaPlaque agarose (0.5%) as indicated.

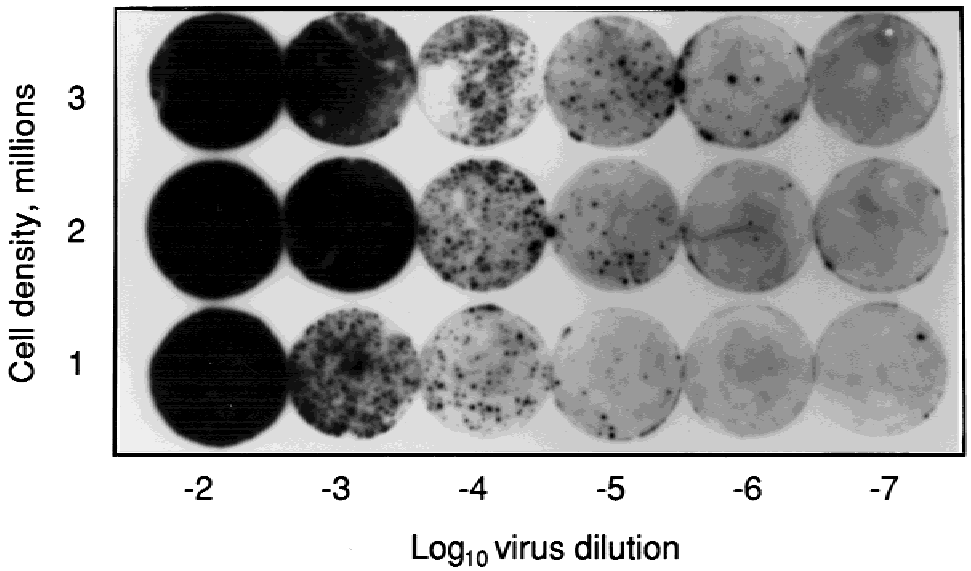

Fig. 6. Development of foci is influenced by cell seeding density. Wells (3 cm diameter) were seeded with 3, 2, or 1×10^6 cells as indicated, and infected one day later with serial dilutions of virus. Cells were fixed at 12 days post-infection.

mate of vge must have been slightly low in this experiment, the infectious dose for ducklings was around 1 vge, as expected [Jilbert et al., 1996].

Comparison of Monoclonal Antibodies

We have used secondary antibody staining in these studies because MAb preS1 proved to be sensitive to iodination with chloramine T (results not shown). Pugh and Simmons [1995] have reported DHBV-specific MAbs which retain activity after iodination, and we therefore assessed the utility of one such antibody (1H.1) in the RIFA. Both pre-S specific MAbs (preS1 and 1H.1) gave satisfactory reactivities (results not shown), and it is therefore likely that 1H.1 could be iodinated and used for direct labelling of DHBV RIFAs.

Measurement of Antiviral Efficacy With the DHBV RIFA

DHBV-infected ducks are used widely as a model system for the study of antihepadnaviral drugs, with reductions in serum viral DNA levels providing a rapid indication of efficacy. While treatment with active nucleoside analogues such as Ganciclovir reduces the viraemia to levels undetectable by dot blot hybridisation [Wang et al., 1991], replication generally relapses rapidly upon cessation of therapy [Dean et al., 1995]. In view of the sensitivity of the DHBV RIFA, we sought to determine the residual level of circulating virus during antiviral therapy as an indication of the true level of antiviral efficacy. Six-week-old ducks, congenitally infected with DHBV, were treated for 4 weeks with placebo (0.85% saline) or Ganciclovir (10 mg/kg, twice daily) by intraperitoneal injection. Serum was collected at intervals, and the titres of infectious virus in the pretreatment- and end of treatment sera were determined by RIFA (Table II). At the commencement of therapy, infectious viraemias varied between individual animals in the range of 0.86 to 5×10^7 RIFU per ml. As expected, serum viral DNA became undetectable by dot blot hybridisation within 4 days (results not shown) [Dean et al., 1995], yet at the end of the 4 week treatment regime both Ganciclovir-treated ducks had residual viraemias exceeding 10^4 RIFU per ml, representing an approximately 3 $\log_{10}$ reduction compared to natural variation in placebo animals within a range of less than 0.5 $\log_{10}$. The ability to detect this residual virus, still well within the dynamic range of the assay, suggests that the RIFA will be especially useful in comparing the level of antiviral effects between highly active drugs or drug combinations in which the serum viral DNA content is suppressed profoundly.

DISCUSSION

The DHBV RIFA fulfills most of the requirements for a useful in vitro infectivity assay. The assay is simple, being a modification of routine plaque assays, and is highly sensitive with a detection limit approaching that of titration in live ducklings. As expected, the dose-response for focus formation is linear, and intra-assay reproducibility is very high (for example, replicates shown in Fig. 1 and the standard deviations between triplicates shown in Fig. 2). The RIFA has three shortcomings, in the extended incubation time of 10 to 12 days plus 3 to 7 days film exposure, the need for freshly prepared primary cells, and the somewhat tedious procedure of indirect staining of large numbers of coverslip cultures. However, primary culture of duck hepatocytes is now straightforward, and the direct labour involved is minimal beyond the preparation of the cell suspension. Most importantly, these shortcomings are balanced by the ability to titrate many samples in a single assay with good reproducibility. This is vital to

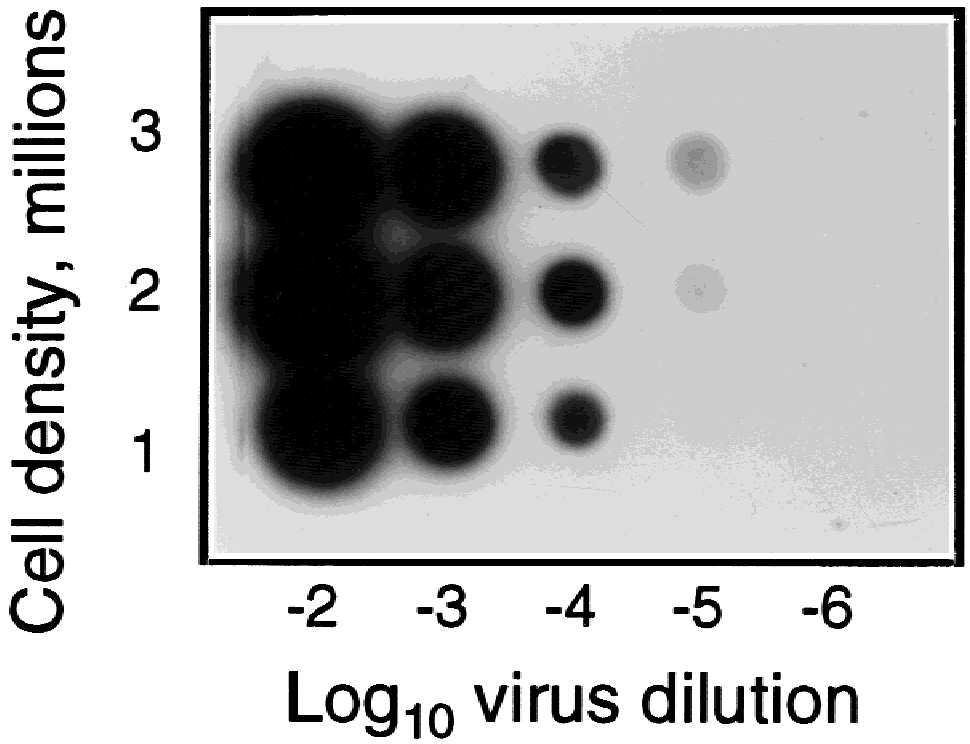

Fig. 7. Detection of viral DNA in infected cultures. Replicates of the cultures shown in Figure 6 were analysed at 12 days post-infection for total viral DNA by dot hybridisation with ^{32}P-labelled probes as described in Materials and Methods. The membrane was exposed to preflashed film for 4 days.

TABLE I. Titration of a DHBV Suspension in Ducklings

Reciprocal dilution[a]	Estimated RIFU/animal	Number of animals[b]	Infected[c]	Not infected
8	0.90	4	4	0
16	0.45	4	3	1
32	0.23	3	3	0
64	0.11	4	2	2
128	0.06	4	0	4
256	0.03	4	0	4

[a]Day-old ducklings were inoculated by intraperitoneal injection with 1 ml of serial dilutions of the virus pool described in Figures 6 and 7 (7.2×10^7 RIFU/ml), beginning from a 10^{-7} dilution.
[b]Four ducklings were inoculated at each dilution. The death was unrelated to the infection and was not scored.
[c]Viraemia was detected at 4 weeks post-inoculation by dot hybridisation with digoxigenin-labelled probes as described in Materials and Methods.

many studies of viral replication or inactivation, and in our hands a single duck yields enough cells for around 240 individual sample wells. In contrast, titration in vivo may take 4 to 5 weeks and requires up to 40 ducks for the accurate determination of the titre of a single sample, especially when the viral DNA content is too low to allow an estimate of the infectious dose. The RIFA is highly suitable for titration of such low-titred samples, but ducklings will remain the method of choice for demonstrating complete removal of DHBV from samples via inactivation, neutralisation, or antiviral effects.

Because of the extended incubation time, the cells require at least one medium change (usually around 5 days post-infection). While we have achieved reasonable results with semi-solid overlays using SeaPlaque agarose, our results indicate that even in liquid media the spread of virus is almost exclusively from infected cells to neighbouring cells. This most likely reflects the mechanism of primary exocytosis in hepatocytes, which occurs exclusively via the basolateral membrane [Hubbard et al., 1989; Ihrke et al., 1993]. The assay is therefore most simply performed with liquid media, with the addition of DMSO required to allow multiple rounds of infection [Pugh and Summers, 1989].

Despite the availability of effective vaccines, infection with hepatitis B will remain a major medical problem for many years, and strategies to improve the vaccine coverage and efficacy and to develop effective antiviral therapies are the focus of extensive research. Furthermore, as a blood-borne virus, HBV poses a major risk of iatrogenic infection and strategies to remove or inactivate HBV in blood-derived products must be strictly validated. The DHBV RIFA has clear use for such studies for the measurement of viral inactivation procedures and antiviral efficacy in cell culture or animal studies, being able to detect very low viral loads. For example, during treatment of DHBV-infected ducks with nucleoside analogues such as ganciclovir or penciclovir/famciclovir the level of viral DNA in serum rapidly falls to or below the limit of detection by hybridisation [Dean et al., 1995; Luscombe et al., 1994; Tsiquaye et al., 1994; Wang et al., 1991], but measurement by RIFA has shown that infectious virus persists

TABLE II. Measurement of Antiviral Effects by RIFA

	Serum RIFU per ml		
Treatment (duck)	Pretherapy	Posttherapy[a]	Log_{10} Reduction[b]
Control (5R)	1.0×10^7	1.2×10^7	−0.08
Control (6R)	5.0×10^7	3.1×10^7	0.21
Control (7R)	8.6×10^6	3.0×10^6	0.46
DHPG[c] (1R)	2.8×10^7	1.5×10^4	3.27
DHPG (26R)	2.2×10^7	5.2×10^4	2.62

[a]Ducks were treated between 6 and 10 weeks of age.
[b]Reduction in infectious viraemia (log_{10} RIFU/ml) during the 4 weeks of treatment.
[c]Ducks were treated with 10 mg/kg DHPG administered twice daily.

for long periods at a titre of around 10^3 to $10^{4.5}$ per ml (Table II) [Luscombe, 1995]. With a sensitivity approaching that of titration in live ducks we expect that the RIFA will find utility in many studies of this model of an important human pathogen.

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